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## **Geranylgeranylacetone inhibits proliferation of human cervical cancer cells in a normal glucose environment**

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### **Abstract**

Oral geranylgeranylacetone (GGA) administration has been used in Asia, particularly in Japan, as an anti-ulcer drug for more than 20 years. Recently, some studies reported that GGA suppresses cell growth and induces apoptosis in cell models of human leukemia, ovarian carcinoma, and colon cancer in vitro. Therefore, the aim of the present study was to determine whether GGA can have a therapeutic effect on human cervical cancer cell line (HeLa cells) in normal and high glucose environments (NG and HG, respectively). The results showed that GGA dose-dependently inhibited proliferation of HeLa cells with cell damage in an NG environment and inhibited those without cell damage in an HG environment. The extent of cell injury was strongest in an NG/GGA (100  $\mu$ M) group 3 days after treatment. DNA fragmentation and flow cytometry analyses revealed that the type of cell death was a combination of necrosis and apoptosis, indicating the potential anti-cancer activity of GGA; however, further studies are needed to investigate the optimal GGA administration route and dose.

### **Introduction**

Oral geranylgeranylacetone administration (GGA; Japanese name, Teprenon) has been used in Asia, particularly in Japan, as an anti-ulcer drug for more than 20 years, and no major adverse effects have been reported. Recently, GGA was shown to activate heat shock protein 70 (HSP70) and exert cytoprotective effects against various stressors in a variety of cells and tissues. In contrast, GGA was shown to suppress cell growth and induce differentiation and apoptosis in human leukemia cell lines (1,2), and it also inhibits lysophosphatidic acid-induced invasion of human ovarian carcinoma cell lines by attenuating activation of rhodopsin (*Rho*) and rat-sarcom-mitogen-activated protein kinase (*Ras-MAPK*) pathways (3,4). Furthermore, a recent study reported that GGA inhibits proliferation of human colon cancer cells by inducing apoptosis and cell cycle arrest (5,6). Taken together, these studies indicated that GGA may have therapeutic potential for treatment of various cancers. Therefore, the aim of the present study was to determine whether GGA can have a therapeutic effect on HeLa cells, which is a human cervical cancer cell line.

## **Materials and Methods**

### ***Cell culture and GGA treatment***

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 200 µg/ml streptomycin, and 200 U/ml penicillin. Experiments were performed using 3–7-day-old cell cultures in DMEM supplemented with either a high glucose (HG; 25 mM) or a normal glucose (NG; 5.6 mM) concentration. GGA (Teprenon; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in ethanol and added to each culture. Control cultures were treated with the vehicle alone. Staurosporine (STS; 4 µM) was added to the cultures, which were then incubated for 8 h, as a positive control of apoptosis. To activate HSP70 expression, the cells were incubated at 42°C for 2 h.

### ***Cell viability assay***

Cell viability was assessed using the trypan blue exclusion test. In total,  $2.0 \times 10^5$  HeLa cells were seeded in dishes (35 mm in diameter) and treated with various GGA concentrations (3, 10, 30, and 100 µM, respectively). As a control, the vehicle alone was assessed. The trypan blue exclusion test was performed by adding equal volumes of a 0.4% trypan blue solution and cell suspension and then evaluated 3 days after treatment. The number of total and live cells was counted under light microscopy 3 days after GGA treatment.

### ***DNA fragmentation assay***

Internucleosomal DNA fragmentation was assessed by a DNA ladder. Briefly, cells were digested in a lysis buffer (5 mM ethylenediaminetetraacetic acid/0.2% sodium dodecyl sulfate/0.2 mg/ml proteinase K/100 mM Tris·HCl; pH 8.5) at 37°C overnight. Chromosomal DNA was analyzed by agarose gel electrophoresis (2%) followed by staining with ethidium bromide, as previously reported (7). DNA fragmentation was also assessed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit with Tetramethylrhodamine red (Roche diagnostics, Basel, Switzerland) for detecting and quantifying apoptosis. The cells were treated with GGA (100 µM) and then assayed 3 days after treatment.

### ***Flow cytometry***

The cells were treated with GGA (100  $\mu$ M) and then analyzed by flow cytometry 3 days after treatment. During early apoptosis, membrane integrity was assessed by staining with annexin V, which binds to exposed membrane phosphatidylserine, an acidic (anionic) phospholipid and one of the earliest features of apoptosis, using the PE Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). Necrotic change was concurrently assessed using 7-amino-actinomycin (7-AAD) staining solution to exclude viable cells with intact membranes.

### ***Statistical analysis***

Statistical analysis was performed using SPSS statistical software (ver. 17; SPSS Inc., Chicago, IL, USA). Analysis of variance was used to determine statistical significance. A  $p$ -value  $<0.05$  was considered statistically significant.

## ***Results***

### ***Cell viability assay***

The effects of various GGA concentrations on cell viability were assessed. The cell concentration increased from  $2.0 \times 10^5$  to  $21.1 \times 10^5$  in HG/control culture and  $11.7 \times 10^5$  in NG/control culture. The total cell count decreased in a dose-dependent manner in GGA-treated cultures under both HG and NG conditions (Fig. 1A). The percentage of live cells decreased in the NG/GGA (100  $\mu$ M) group in a dose-dependent manner, whereas there was no remarkable change in the HG/GGA group. The reduction was remarkable in the NG/GGA (100  $\mu$ M) group (Fig. 1B), and cell toxicity in that group was observed only 3 days after treatment (Fig. 1C). The percentage of live cells in the NG /GGA (100  $\mu$ M) group reduced to 44.0% 3 days after treatment, whereas that under other conditions was approximately 90%. Microscopic changes in morphological features are shown in Fig. 2.

### ***DNA fragmentation assay***

The DNA fragmentation assay was performed to detect whether cell death was caused by apoptosis or necrosis. As shown in Fig. 3A, DNA laddering was not evoked in the NG/GGA (100  $\mu$ M) group, but it was evoked with exposure to 4  $\mu$ M STS for 8 h, the positive control of apoptosis. The TUNEL assay results were positive in the NG/GGA (100  $\mu$ M) group (Fig. 3B).

### ***Flow cytometry***

Late apoptotic or dead cells were measured by PE Annexin V and 7-AAD staining following NG/GGA (100  $\mu$ M) and control treatments. As shown in Fig. 4, the population of late apoptotic/dead cell increased after GGA treatment in the NG environment. The percentage of late apoptotic/dead cells was  $81.4 \pm 9.1\%$  in the NG/GGA (100  $\mu$ M) group and  $12.34 \pm 4.60\%$  in the NG/control group. There was no significant change in the HG/GGA (100  $\mu$ M) group, and the percentage of early apoptotic cells in each group did not significantly change (Fig. 4).

### **DISCUSSION**

The anti-ulcer mechanism of GGA can reportedly enhance the mucous and mucosal barrier, thereby protecting the stomach against its own secretions. In addition to this mechanism, in 1996, Hirakawa et al. reported that GGA can nontoxically induce HSP70 expression and protect cultured gastric mucosal cells (8). Several studies revealed that low-dose GGA induced HSP70 expression and had protective effects in glaucoma (9), cerebellar infarction (10), liver transplantation (11), cardiac ischemia/reperfusion injury (12), polyglutamine disease (13), and pulmonary fibrosis (14) in animals.

Recently, inhibitors of isoprenyl compounds such as farnesol, geranylgeraniol, and geranylgeranoic acid were shown to induce apoptotic cell death. Some isoprenoids with strong anti-cancer effects, such as docetaxel and paclitaxel (15-20), are currently available for clinical use. The chemical structure of GGA is similar to that of geranylgeranyl pyrophosphate, which is a component of the Rho metabolic pathway and is essential for Rho geranylgeranylation. Furthermore, GGA has been shown to induce differentiation and apoptosis in human leukemia cell lines through modulation of small G protein activation (1,2) and inhibit ovarian cancer progression through Rho inhibition and Ras-MAPK activity (3,4). More recently, GGA was reported to inhibit human colon cancer cell proliferation through induction of apoptosis and cell cycle arrest (5). Taken together, these studies indicate that GGA has potential as an anti-cancer drug. Therefore, we evaluated the effects of GGA using HeLa cells, as an in vitro model of human cervical cancer.

In this study, we employed a cell viability assay to evaluate the influence of GGA concentration on proliferation of HeLa cells, which are generally cultured in HG DMEM (450mg/dL; 25mM); however, serum NG content in humans is approximately 100 mg/dl (5.6 mM). Therefore, we examined both HG DMEM (25 mM) and NG DMEM (5.6 mM). The medium was changed with GGA treatment. The number of

HeLa cells in the HG and NG control groups increased by approximately 10-fold and 5-fold, respectively, after 3 days. GGA dose dependently inhibited proliferation of HeLa cells in both the HG and NG groups. The total cell count of the HG/GGA and NG/GGA (100  $\mu$ M) groups 3 days after treatment was approximately half of each control group.

The trypan blue exclusion test was performed to investigate cell damage caused by GGA. The percentage of live cells dose-dependently decreased in the NG group, whereas there was no change in the HG group. The percentage of live cells was clearly decreased in the NG/GGA (100  $\mu$ M) group 3 days after GGA treatment. These results indicated that GGA dose-dependently inhibited proliferation of damaged HeLa cells in an NG environment and inhibited proliferation of undamaged cell in a HG environment. Notably, cell injury was most pronounced in the NG/GGA (100  $\mu$ M) group 3 days after treatment.

Cell death can be distinguished by apoptotic and necrotic events. In this study, the type of cell death was examined by flow cytometry. HeLa cells that were treated by HG/GGA and NG /GGA (100  $\mu$ M) for 3 days were stained with PE Annexin V and 7-AAD to detect early apoptosis and necrosis, respectively. The percentage of late apoptotic/dead cells was significantly increased in the NG/GGA (100  $\mu$ M) group compared with other groups. The percentage of early apoptotic cells did not significantly increase. These results were in accordance with those of the cell viability assay. The DNA fragmentation assay was also used to examine the type of cell death induced by GGA. Our results showed no DNA fragmentation in NG/GGA (100  $\mu$ M) group 3 days after treatment, in which the most significant cell injury was observed in the cell viability assay. In the STS group, a clear DNA fragmentation was observed, which was used as a positive control of apoptosis. In contrast, apoptotic change was detected in the NG/GGA (100  $\mu$ M) group by the TUNEL assay. These results indicated that cell death and apoptotic changes were induced in HeLa cells after NG/GGA (100  $\mu$ M) treatment for 3 days, but typical apoptosis was not induced by STS. GGA treatment remarkably disrupted cellular adhesion, but it was maintained in the STS group. Previous studies have shown that effects of in vitro GGA concentrations on cancer cells vary approximately from 1  $\mu$ M (2,21) to 100  $\mu$ M (1,4,22), which were supported by our results.

An HG dose combined with GGA has cytostatic and cytotoxic effects on HeLa cells in an NG environment, but it remains unknown whether an HG dose combined with GGA induced HSP70 expression in HeLa cells. Western blot analysis revealed that HSP70 was induced in HeLa cells incubated in NG/GGA (100  $\mu$ M) for 1 day (data not shown). HSP induction is upregulated in cancer cells and can be a factor of thermotolerance and

resistance to thermotherapy by cancer cells (23). HSP70 was upregulated in HeLa cells following HG/GGA treatment, but it was ineffective against cytotoxicity and GGA-induced cell death.

Our results showed that HG/GGA treatment was cytotoxic to HeLa cells in an NG environment, indicating the potential of GGA as an anti-cancer drug. Because of hypoxia, hypoglycemia, and acidemia in the hypovascular environment of solid cancers, cells become resistant to anti-cancer drugs such as etoposide, doxorubicin, and camptothecin (24-26). However, further studies are required to examine the effects of hypoxic and hypoglycemic environments on cancer cell proliferation. Furthermore, a pharmacokinetic study revealed that the serum concentration of a usual therapeutic dosage after GGA administration was 1–10  $\mu\text{M}$  (27). Therefore, the route of administration should be considered because serum GGA concentrations cannot increase to 100  $\mu\text{M}$  by oral administration. Therefore, studies of actual GAA therapeutic concentrations in cancer tissue should be examined using animal models. We examined the effect of GGA on HeLa cells in vitro and found that GGA exerted a cytostatic effect in a dose-dependent manner in NG and HG environments, and it had cytotoxic effects in an NG environment. Apoptosis was induced in the damaged cells. The results of the present study indicate that GGA is a potentially useful anti-cancer drug, but further studies are required to validate these results.

### **Acknowledgements**

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### **Figure legends**

Fig. 1

(A) Total cell count and (B) Percentage of live cells 3 days after treatment with Geranylgeranylacetone (GGA) (3, 10, 30, and 100  $\mu$ M). (C) Changes in the percentage of live cells after 3 days in control and high glucose (HG) and normal glucose (NG) GGA (100  $\mu$ M)-treated groups. All data are presented as means  $\pm$  SEM (vertical bar) of 5 observations. \* $p$  < 0.05 vs. corresponding control

Fig. 2

Microscopic changes in the morphological features of HeLa cells 2 days after Geranylgeranylacetone treatment day 3 (magnification,  $\times 400$ ) (A) High glucose (HG)/control; (B) HG/(GGA) (100  $\mu$ M); (C) Normal glucose (NG)/control; (D) NG/GGA (100  $\mu$ M); and (E) Staurosporine (STS)

Fig.3

(A) DNA laddering and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick and labeling (TUNEL) 3 days after GGA (100  $\mu$ M) treatment. (B) High glucose (HG)/control; (C), HG/GGA (100  $\mu$ M); (D), Normal glucose (NG)/control; (E), Normal glucose (NG)/GGA (100  $\mu$ M); and (F) Staurosporine (STS)

Fig. 4

Cell apoptosis was detected by annexin staining (x-axis), and late apoptosis or necrosis was determined by 7-AAD staining (y-axis)

(A) Representative results (B) The percentage of early apoptotic cells and late apoptotic or necrotic cells are shown. All data are presented as means  $\pm$  SDM (vertical bar) of 3 observations. \* $p$  < 0.05 vs. corresponding control



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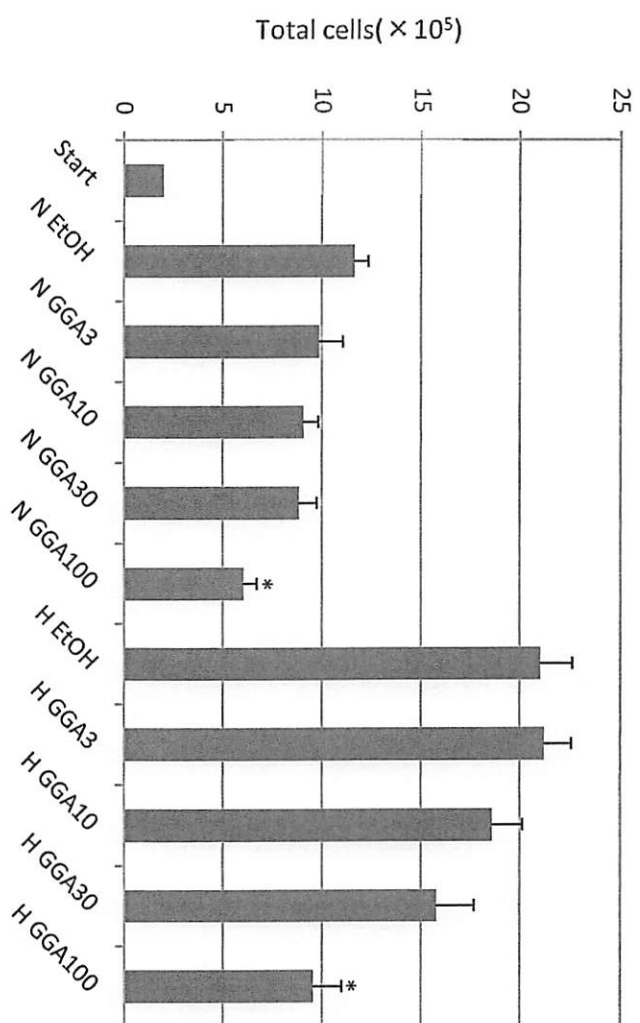
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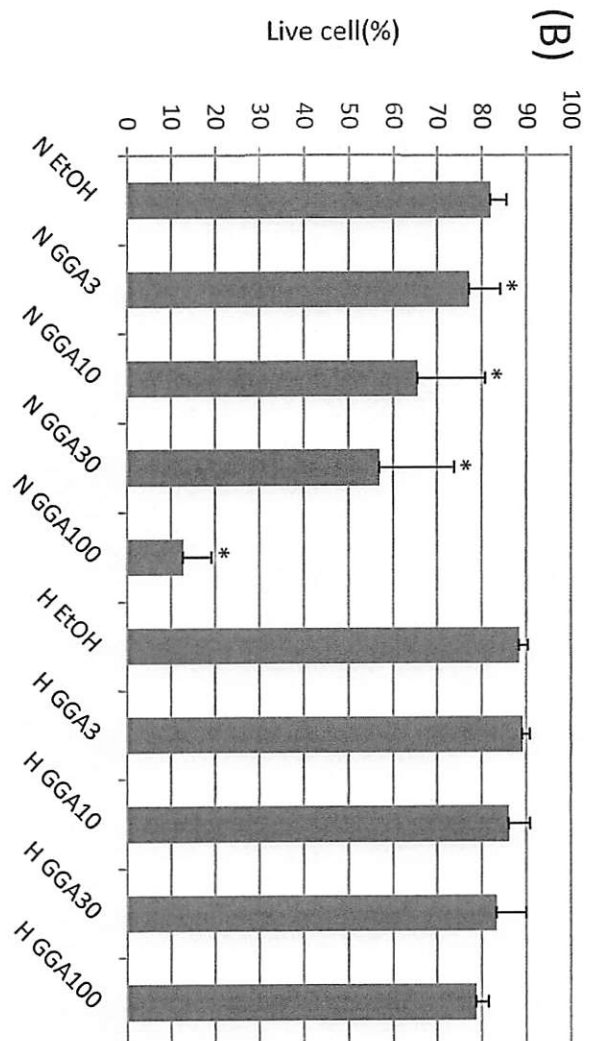
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Fig. 1 (A)



(B)



(c)

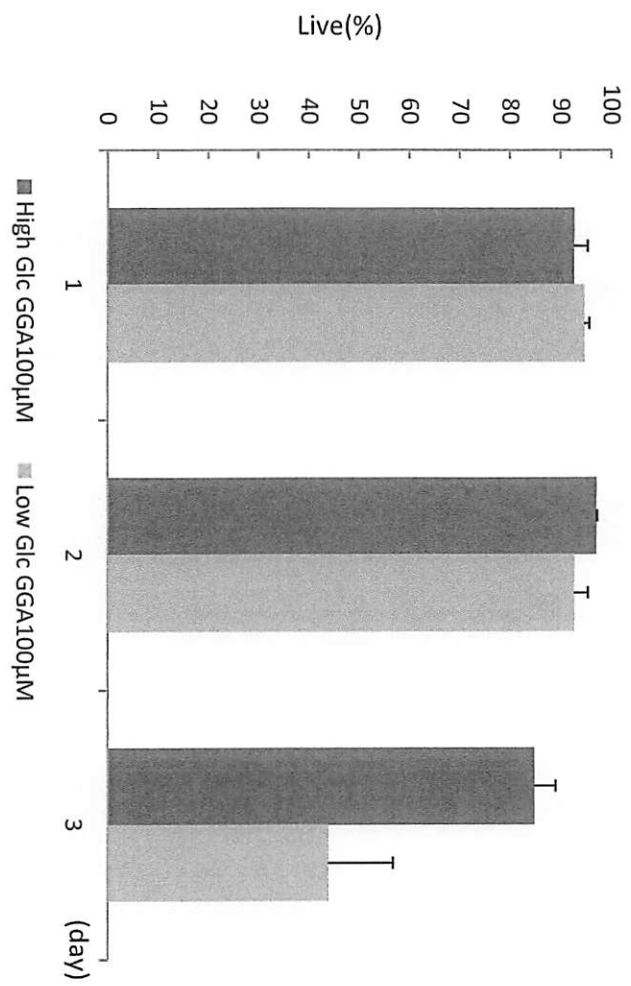
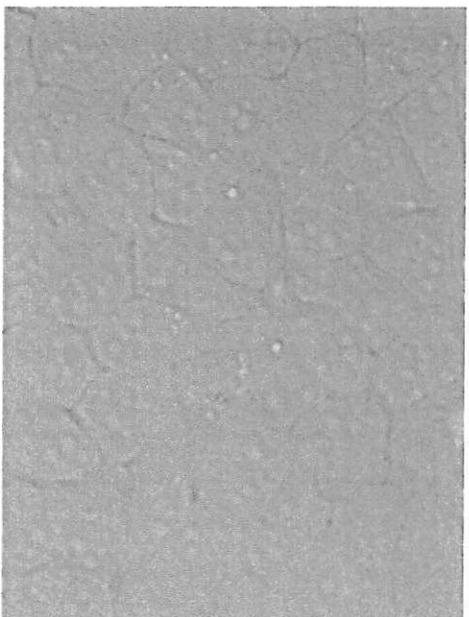
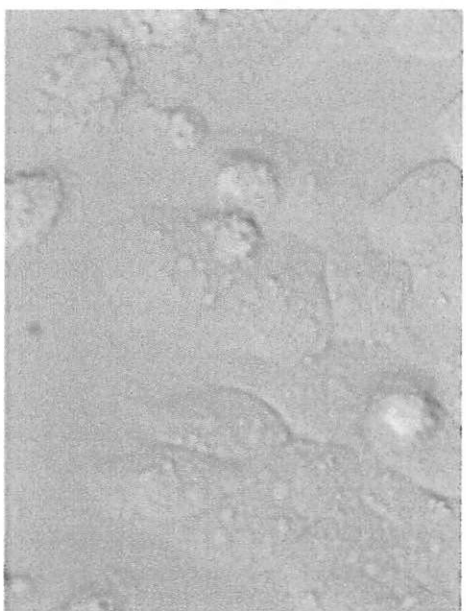


Fig2. (A)



(B)



(C)



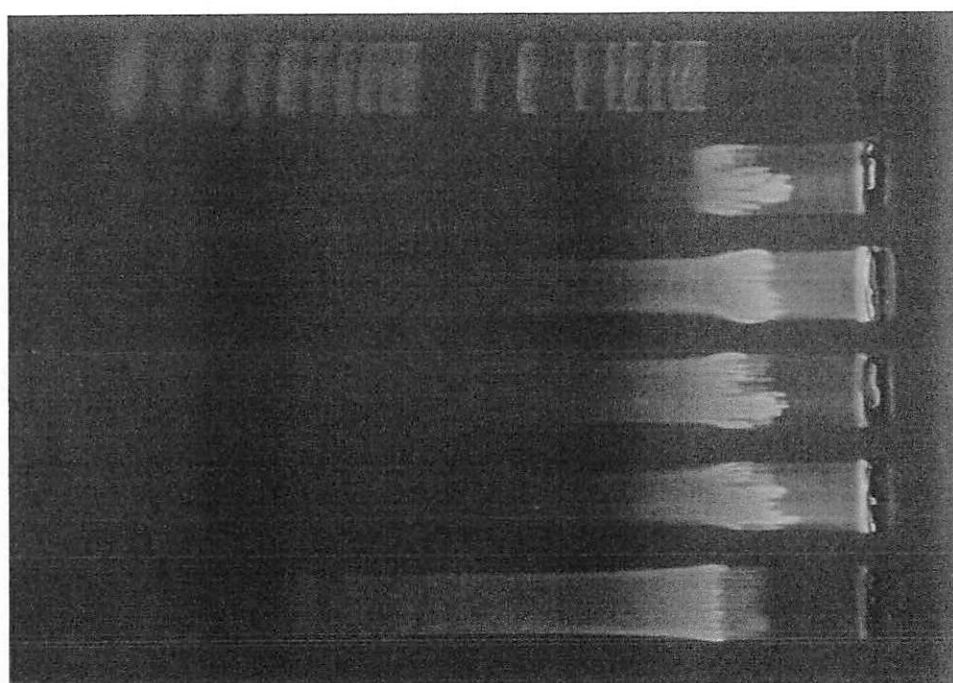
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(E)



Fig. 3 (A)



100bp Marker

HGlc control

HGlc GGA100 $\mu$ M

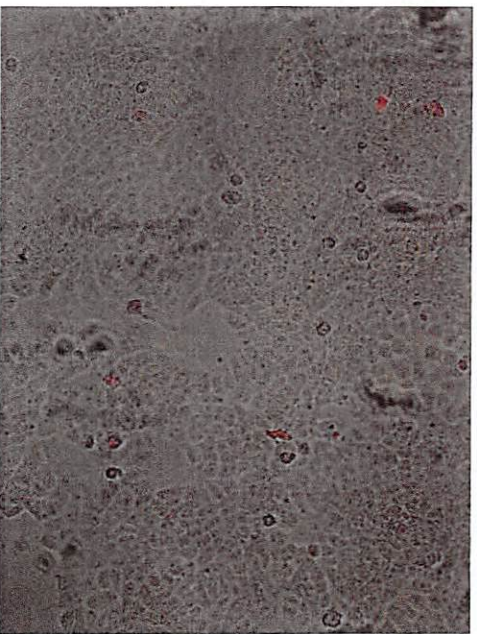
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NGlc GGA100 $\mu$ M

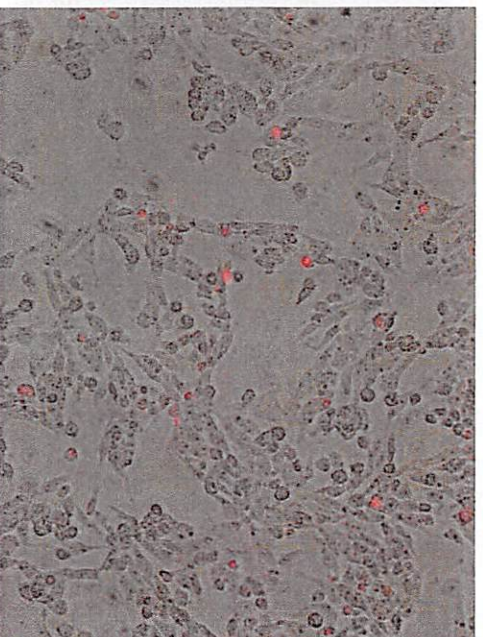
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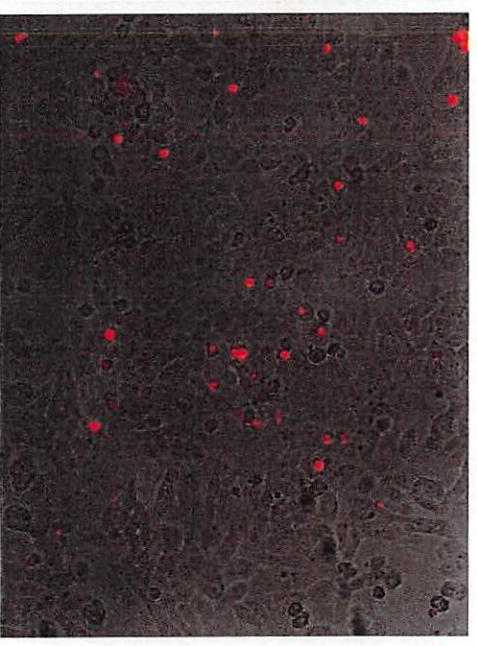
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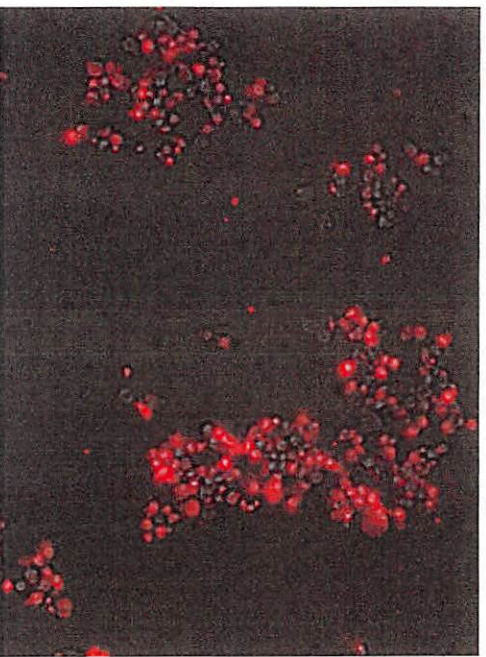
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(D)



(E)



(F)

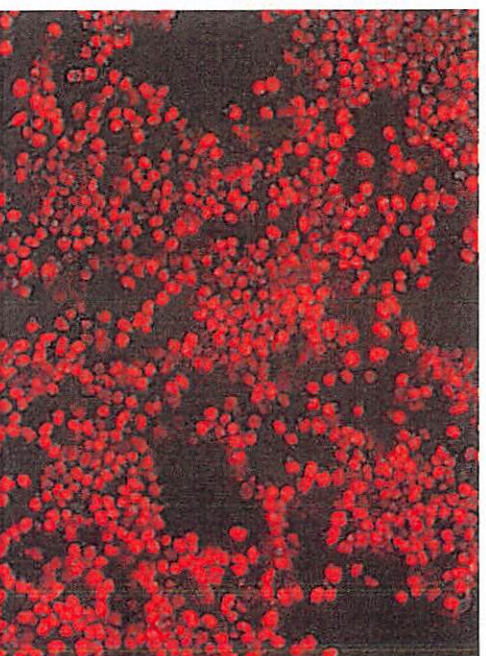
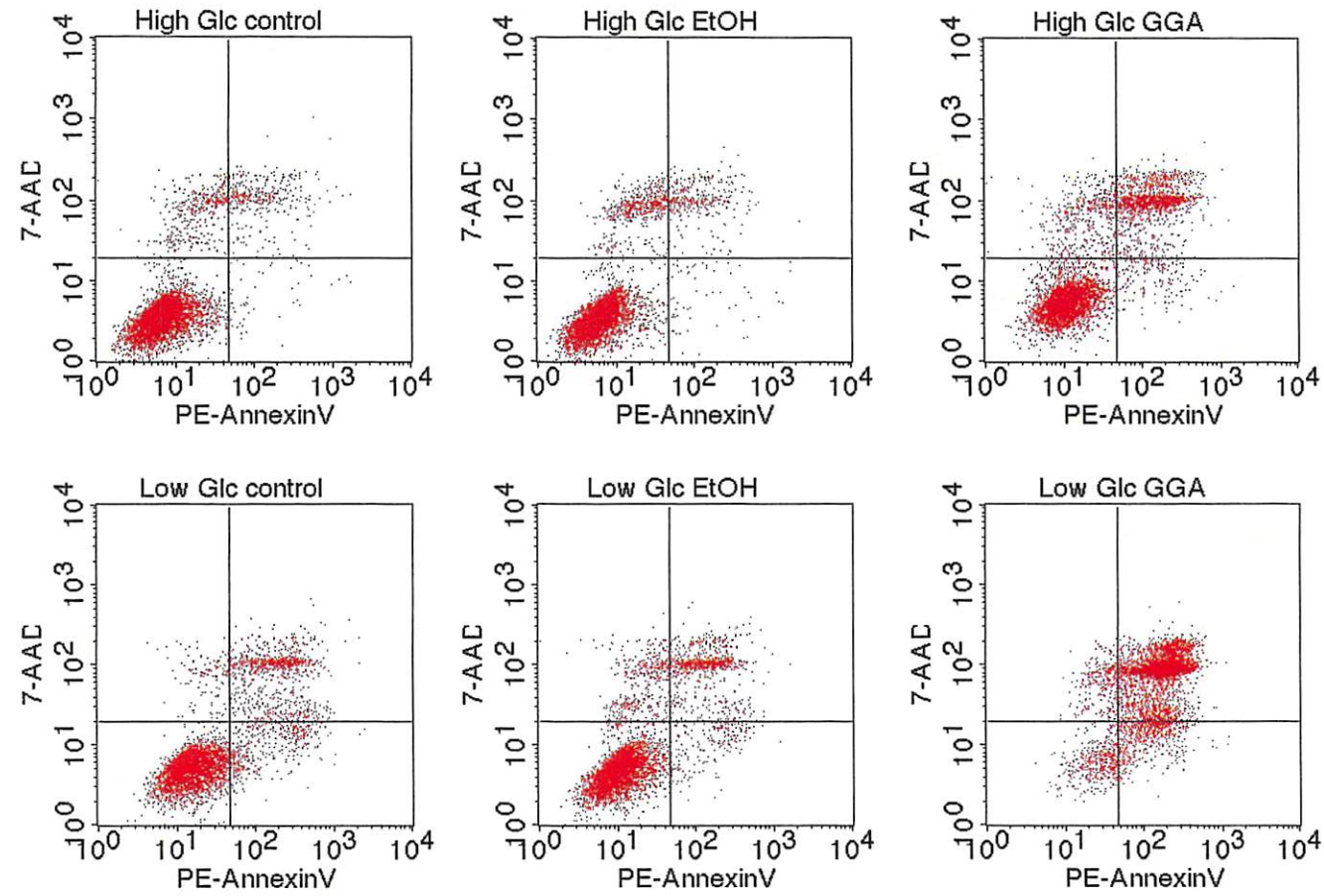




Fig.4 (A)



(B)

